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Genetic diversity among Spanish pea (*Pisum sativum* L.) landraces, pea cultivars and the World *Pisum* sp. core collection assessed by retrotransposon-based insertion polymorphisms (RBIPs)

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Abstract

A total of 122 accessions of different wild and cultivated *Pisum* sp. were analysed using retrotransposon-based insertion polymorphisms (RBIP) markers. The *Pisum* materials included wild and cultivated (landraces and cultivars) materials from the World core collection of the John Innes Centre (JI) representing all generally recognized *Pisum* taxa, landraces materials from the Spanish core collection, and commercial pea cultivars largely sown in Spain. The overall polymorphism detected by RBIP marker was high and all accessions, except two pairs, could be distinguished by their marker pattern. Principal component and phylogenetic analyses clearly discriminated *P. fulvum* and *P. abyssinicum* samples from both each other and *P. sativum*, while *P. elatius* and *P. humile* samples were scattered among the other taxa clusters, supporting the existence of three well defined taxa in the genus *Pisum* (*P. abyssinicum*, *P. fulvum* and *P. sativum*). These results also suggest that the Spanish pea core collection of landraces maintains a relatively high variability which is only partially represented in cultivars generally sown in Spain. Thus, Spanish landraces are still a source of genetic variability for breeding new pea cultivars.

Additional key words: genetic resources, *Pisum abyssinicum*, *Pisum fulvum*, *Pisum sativum*, RBIP markers.

Resumen

Diversidad genética en variedades locales y cultivares españoles de guisante (*Pisum sativum* L.) y en la colección nuclear mundial de *Pisum* estimada mediante polimorfismo de inserción de retrotransposones (RBIP)

Se ha estudiado un total de 122 accesiones silvestres y cultivadas de *Pisum* sp. usando marcadores basados en polimorfismos de inserción de retrotransposones (RBIP). Las accesiones de *Pisum* incluyen materiales silvestres y cultivados (cultivares y variedades locales) de la colección nuclear mundial del John Innes Centre (JI) representando a todos los taxones generalmente reconocidos de *Pisum*, variedades locales de la colección nuclear española, y por último algunas variedades comerciales de guisante ampliamente cultivadas en España. Para el análisis genético se usaron 18 loci RBIP. El polimorfismo general detectado con los marcadores RBIP fue alto y todas las muestras, excepto dos pares, pudieron ser identificadas por un patrón particular de marcadores. Análisis de componentes principales y filogenéticos discriminaron claramente *P. fulvum* y *P. abyssinicum* entre ellas y de *P. sativum*, mientras que las muestras de *P. humile* y *P. elatius* se mezclaban con las de otros taxones en distintos grupos. Esto apoya la existencia de tres especies en el género *Pisum* (*P. abyssinicum, P. fulvum* y *P. sativum*). Los resultados indican que la colección nuclear española de guisante mantiene una variabilidad relativamente elevada que está sólo parcialmente representada en los cultivares generalmente sembrados en España. Por tanto, las variedades locales españolas representan aún una fuente de variabilidad genética para la mejora de nuevos cultivares.

Palabras clave adicionales: marcadores RBIP, *Pisum abyssinicum*, *Pisum fulvum*, *Pisum sativum*, recursos genéticos.

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Abbreviations used: IRAP (inter-retrotransposon amplified polymorphism), ISSR (inter simple sequence repeats), JI (John Innes Centre), PCA (principal component analysis), RBIP (retrotransposon-based insertion polymorphisms), REMAP (retrotransposon-microsatellite amplified polymorphism), SSAP (sequence-specific amplification polymorphisms), ZP (ITACyL collection).

Introduction

Pea (*Pisum sativum* L.) is a major cool season legume crop for human consumption, as dry seeds or as vegetable, and for feeding livestock. Pea was also one of the first domesticated crops in the Old World and one of the first genetic research materials. The modern gene pool of cultivated *Pisum* is diverse, reflecting this early domestication and subsequent widespread cultivation. However, in spite of the extensive phenotypic and genetic variability, existing taxonomic classifications are confusing (Vershinin *et al.,* 2003; Kosterin and Bogdanova, 2008). In addition to *P. sativum*, two other species are generally recognized within the genus *Pisum*, the wild *Pisum fulvum* Sibth. et Smith., which is almost reproductively isolated from *P. sativum*, and *Pisum abyssinicum* A. Br. represented by cultivated and some wild forms from South Arabia and Ethiopia. Other taxa once considered as species are in fact *sensu lato* representatives of *P. sativum*. Some of them are presently considered as subspecies, although the subspecies concept in the case of the pea remains quite vague, and according plastid, mitochondrial and nuclear markers all wild forms of *P. sativum* would better be considered within a fuzzy paraphyletic subspecies *P. sativum* ssp. *elatius* (Bieb.) Schmalh. *sensu lato* (Kosterin and Bogdanova, 2008). A similar phylogenetic organization of taxa was previously described by Maxted and Ambrose (2001) in which three species were recognized (*P. abyssinicum*, *P. fulvum* and *P. sativum* with two subspecies ssp. *sativum* and ssp *elatius* (Bieb.) Aschers. & Graebn., considering *P. humile* Boissier and Noe as a variety of *P. sativum* spp. *elatius*). According to plastid, mitochondrial and nuclear sequences (*rbcL*, *coxI* and *SCA*, respectively) Kosterin *et al.* (2010) pointed to the lineage B of *P. sativum* ssp. *elatius* as the origin of the cultivated *P. sativum*. A study of the genetic structure and evolutionary history of *Pisum* based on retrotransposon sequence-specific amplification polymorphisms (SSAP) revealed high polymorphism in all species, except *P. abyssinicum*. The results indicated a high contribution of recombination between multiple ancestral lineages compared to transposition within lineages, suggesting that the two independently domesticated pea species, *P. abyssinicum* and *P. sativum*, arose independently in contrasting ways via the common processes of hybridization, introgression, and selection (Vershinin *et al.*, 2003).

Retrotransposons are ubiquitous in plant genomes and they vary in copy number and chromosomal location within and between species, and play significant roles in genome evolution (Flavell *et al*., 1992; Baucom *et al.*, 2009; Hawkins *et al.*, 2009). Particular retrotransposon families can vary greatly in abundance and chromosomal location even between closely related species (Pearce *et al.,* 1996; Kubis *et al.,* 1998; Hill *et al.,* 2005), or contribute to novel satellite repeats (Macas *et al.,* 2009). Because of this ubiquity and diversity, retrotransposons based markers are powerful tools for the assessment of genetic diversity, and have shown their usefulness as genetic markers and in biodiversity and phylogenetic analyses (Ellis *et al.,* 1998; Flavell *et al.,* 1998; Vershinin *et al.,* 2003; Jing *et al.,* 2007; Martín-Sanz *et al.,* 2007; Agarwal *et al.,* 2008; Tam *et al.,* 2009; Jing *et al.,* 2010).

Several types of genetic markers have been derived from retrotransposons, including retrotransposon-based insertion polymorphisms (RBIP), inter-retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP), and SSAP (Waugh *et al.*, 1997; Syed and Flavell, 2006); and they have been used in genetic analyses such as gene mapping (Ellis *et al.,* 1998), genotyping, and pea cultivar fingerprinting (Smýkal, 2006). RBIP markers detect presence or absence of individual retrotransposon insertions in the genome; the method requires flanking sequence information for primer design and yields co-dominant markers, where the different allelic states at a locus can be revealed (Flavell *et al.,* 1998). RBIP markers have been recently used for both broad diversity analysis and variety discrimination in pea (Smýkal *et al.,* 2008b; Jing *et al.,* 2010) and have proved to be the most robust and easy to score retrotransposon-based marker method in comparison to IRAP and other marker systems (Smýkal *et al.,* 2008a).

The estimation of the genetic variability within pea collections and the relationships between accessions using molecular markers has been carried out in numerous works. Recently, evaluations of genetic diversity among European pea materials using isozyme, protein and PCR markers and among Spanish materials using inter simple sequence repeats (ISSR) markers have been published (Baranger *et al.,* 2004; Lázaro and Aguinagalde, 2006). Zong *et al.* (2009) analyzed two very wide collections of Chinese and world accessions (this later collection included some wild materials), respectively, using microsatellite markers. They found that the genetic diversity of *P. sativum* within China appears to be quite different to that detected in the global gene

pool, these genetically distinct gene pools within domestic field pea has significant implications in broadening the available variability for further genetic improvement.

Here we described the use of RBIP markers in the analysis of genetic variability and relationships of the Spanish *P. sativum* landrace core collection and pea cultivars currently sown in Spain in relation to a representative set of the *Pisum* World core collection provide by the John Innes Centre, which includes wild and cultivated accessions. The results obtained will contribute to gain a better knowledge of *Pisum* resources genetic variability and be useful for future breeding purposes to improve pea cultivars.

Material and methods

The *Pisum* accessions used are summarized in Table 1. They included wild and cultivated materials, some of them are included in the World core collection of the John Innes Centre (JI), another set of materials represent the Spanish core collection of landraces (Caminero *et al.,* 2001; Ramos, 2003) and traditional varieties (ZP) conserved in the Spanish gene bank, and finally some commercial cultivars largely cultivated in Spain, currently or in the past. Spanish landraces were collected by the Plant Genetic Resource Center (INIA, Spain) from 1971 to 2000 in pea-growing areas with different agroclimatic conditions. These materials have been traditionally cultivated by local farmers in conventional and organic farms. The JI accessions included in this study coincide with accessions included in previous works (Jing *et al.,* 2005, 2007); this JI collection represents the diversity of the genus *Pisum* (Jing *et al.,* 2005). All materials will be referred as accessions in this paper. A total of 122 accessions were analyzed. A summary on the species and the type of material (landrace, cultivar) is shown in Table 1, additional information on their collection numbers, names, origin, etc., is compiled in Appendix 1. Irrespective of the mainly accepted taxonomic classification of *Pisum* species and subspecies (Maxted and Ambrose, 2001), the binomial nomenclature will be used in this work for simplicity.

Genomic DNA was isolated from young leaf tissue using the Quiagen (Valencia, CA) Dneasy 96 plant kit method according manufacturer recommendations. A single individual per accession was analyzed. The RBIP technique was performed as described by Flavell *et al.* (1998), using as primers the flanking sequences of 25 insertions of the retrotransposon *PDR1* defined by Jing *et al.* (2005). A total of 25 RBIP markers were assayed, but monomorphic and those amplifying multiple bands were not considered. The primer sequences and size of the 18 RBIP loci scored is summarized in Appendix 2. Each RBIP was considered as a locus, defining one or more alleles depending of the amplicon fragment size, or the absence of any PCR product corresponding to primer site mutation (Jing *et al.,* 2005) (Fig. 1). Expec-

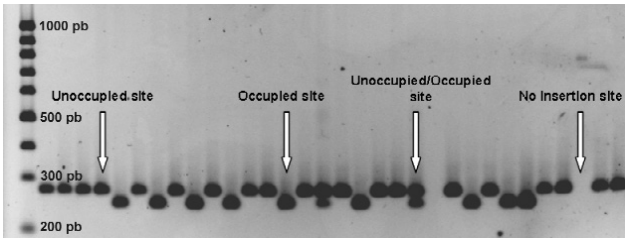

Figure 1. Agarose gel (1.5%) showing three different alleles of the RBIP locus *281x1*. Numbers indicate the reference size markers in base pairs.

Table 1. *Pisum* materials[a] used in the retrotransposon-based insertion polymorphisms (RBIP) analysis

Species	World collection	Spanish landrace collection	Cultivars	Others	Total
P. sativum					
ssp. *sativum*	22	43	27	1	93
ssp. *elatius*	12				12
ssp. *humile*	2				2
P. fulvum	10				10
P. abyssinicum	1			4	5
Total	47	43	27	5	122

[a] Figures indicate the number of accessions analyzed.

Table 2. Average of expected heterozygosity (H_e) over loci

Collection	Average	Standard deviation	Maximum value (locus)	Minimum value
Whole collection[a]	0.451	0.167	0.640 (*45-x31*)	0.079 (*2385-x64*)
P. fulvum	0.144	0.220	0.580 (*95-x2*)	0.0 (12 loci)
P. elatius	0.367	0.224	0.653 (*399-80-46*)	0.0 (3 loci)
P. sativum Wc[b]	0.370	0.202	0.628 (*64-x45*)	0.0 (3 loci)
P. sativum Sc[c]	0.373	0.208	0.623 (*281-x1*)	0.0 (2 loci)
P. sativum cc[d]	0.222	0.220	0.590 (*45-x29*)	0.0 (6 loci)

[a] Including *P. abyssinicum* and *P. elatius*. These two species were not included in the subset analyses due to the low number of accessions. [b] Wc: World collection. [c] Sc: Spanish landrace collection. [d] cc: commercial cultivars.

ted heterozygosity was estimated as $H_e = 1 - \Sigma p_i^2$, were p_i are the allelic frequencies. Shared allele distance (Chakraborty and Jin, 1993) was calculated from the proportion of shared alleles P_{AS} as $D_{AS} = 1 - P_{AS}$. Nei distance was also calculated as 1-I_S. Both distances were calculated by the program MSAT2 (http://hpgl.stanford.edu/projects/microsat/). Bootstrap was carried out, and Neighbour-Joining and UPGMA methods were used for generating trees. Principal component analysis was performed using «Proportion-of-shared-alleles distance» genetic distance.

Results

A total of 122 accessions were analyzed using 18 RBIP loci. A total of 56 alleles were observed in the 18 loci (2 to 4 alleles per locus). As expected in highly self-pollinated materials the homozygosity was predominant, only 13 cases of heterozygosity were detected in the 2,196 accession × loci combinations, approximately a 0.6% of observed heterozygosity which agree with the predominantly self-pollination mating system of *Pisum*. Twelve accessions and seven loci showed at least a case of heterozygosity. The RBIP alleles used indicated a relatively high level of polymorphism. The expected heterozygosity (H_e) for the whole set of accessions and loci was 0.658, the average heterozygosity among loci was 0.451 ranging from 0.079 (locus *2385-x64*) and 0.640 (locus *45-x31*). For those set of accessions represented by at least 10 accessions the heterozygosity values are indicated in Table 2. The levels of polymorphism maintained within the pea World collection, the Spanish landrace collection and *P. elatius* were similar (H_e approximately 0.370) while the set of cultivars showed a significantly ($p < 0.05$) lower average value (0.222).

This high polymorphism of RBIP markers allowed identifying all accessions by a RBIP pattern combination, except *P. fulvum* accessions JI-2519 from JI-2544 and cultivars Lucy from Messire (both French commercial cultivars), respectively. Thus RBIPs are suitable markers for the identification of pea materials. Some species-specific alleles due to retrotransposon insertion were observed in *P. abyssinicum* (loci *2385-x64* and *95-x19*) and *P. sativum* (loci *45-x31, Birte-x34*, and *281-x1*). Since the probability of detecting rare alleles increases as sample size increases, it is possible that the species-specific RBIP alleles observed in the most represented sample of *P. sativum* (93 accessions) represent rare *Pisum* alleles, but in a sample of only five *P. abyssinicum* accessions those alleles most be «true» species-specific alleles, frequent in a species but rare or absent in related species.

The average distances within and between taxa are shown in Table 3. On the basis of RBIP polymorphisms

Table 3. Average distances[a] within and between *Pisum* species (standard deviation)

	P. fulvum	***P. abyssinicum***	***P. humile***	***P. elatius***	***P. sativum***
P. fulvum	0.172 (0.083)				
P. abyssinicum	0.570 (0.096)	0.307 (0.161)			
P. humile	0.460 (0.052)	0.561 (0.076)	0.426 (0.091)		
P. elatius	0.556 (0.073)	0.562 (0.090)	0.444 (0.133)	0.389 (0.124)	
P. sativum	0.640 (0.094)	0.647 (0.098)	0.498 (0.120)	0.485 (0.139)	0.388 (0.130)

[a] Proportion-of-shared-alleles distance.

and H_e, *P. fulvum* seemed to be the less diverse taxon, while *P. elatius* and *P. sativum* showed similar level of internal diversity, and *P. abyssinicum* was lower than these two taxa. The number of accession of *P. humile* was too low in our study to draw valid conclusions. On the other hand, *P. fulvum* and *P. abyssinicum* showed the greatest and similar average distances to *P. sativum*.

Clustering methods showed low bootstrap confidence values for tree nodes. Although these values were too low to be significant, the two distances used (proportion-of-shared-alleles and Nei) and two clustering methods (Neighbour-Joining and UPGMA) generated very similar or identical topologies (data not shown), which conferred robustness to the results. The shown data were obtained with the proportion-of-shared-alleles distance and Neighbour-Joining method. The unrooted tree (Fig. 2) showed that *P. fulvum* formed a well differentiated cluster (1 in Fig. 2); *Pisum abyssinicum* formed a second cluster with some *P. elatius* accessions (2). The remaining *P. elatius* and *P. fulvum* accessions were mainly grouped with pea landraces. All the pea cultivars were grouped in a big cluster (3) with some pea landraces. Pea cultivars included in this group belong to two sets of accessions, to materials sown in Spain and to cultivars included in the JI collection (JI-321 is cultivar Alaska from Canada, JI-399 is Cenia-The Netherlands, JI435 is Wisconsin Perfection-USA, JI-516 is Maro-UK, and JI-113 is an unnamed cultivar from Russia). The remaining pea landraces were scattered in several small clusters irrespective their origin, Spanish landraces or landraces from JI collection.

Principal component analysis (PCA) also clearly discriminated between *P. fulvum* and *P. abyssinicum* and these two taxa from the remaining *Pisum* accessions (Fig. 3). Each of the three first components explained percentages of the total variance higher than 10%

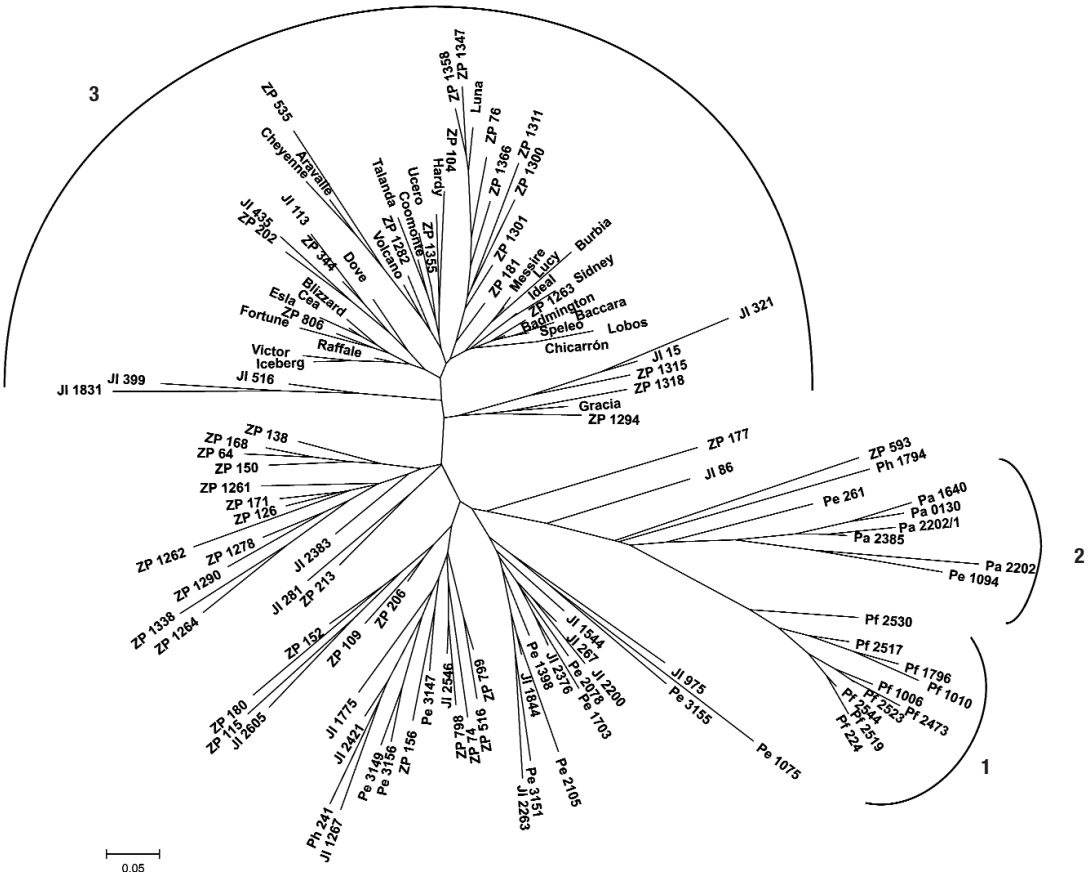

Figure 2. Neighbour joining unrooted tree for *Pisum* accessions deduced from 18 RBIP retrotransposon markers. Names indicate the accession register as follow: Pa, *P. abyssinicum;* Pe, *P. elatius;* Pf, *P. fulvum,* Ph, *P. humile* from the John Innes World Core Collection (JI), all other accessions are registered as *P. sativum.* Numbers indicate the register number at the JI and the ITACyL (ZP) collections, respectively. Accessions indicated with names are cultivars from the ZP collection. 1, 2 and 3 indicate the clusters in which *P. fulvum, P. abyssinicum* and the pea cultivars are grouped, respectively. Branch length units are shown at the bottom.

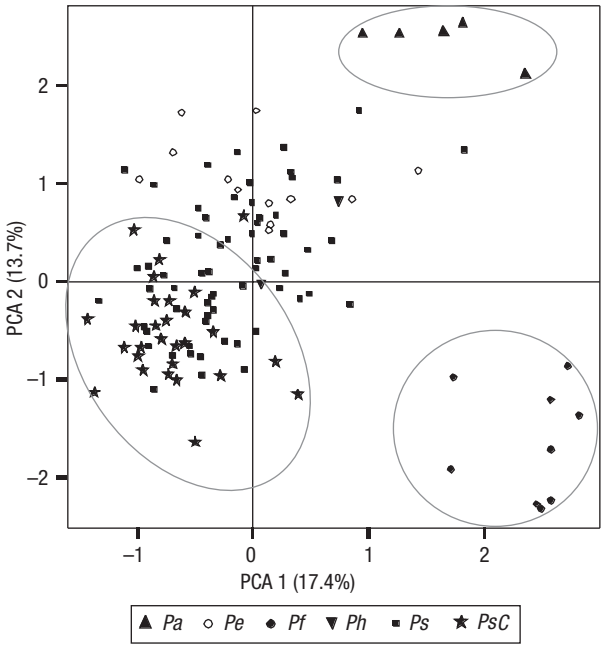

Figure 3. Plot of the principal component analysis. Abbreviations as in Figure 2. Ps, *Pisum sativum* landraces and traditional materials; PsC, *pea cultivars. P. fulvum, P. abyssinicum* and pea cultivars are highlighted within circles.

(17.4, 13.7 and 10.7, respectively; 41.7% accumulated). The first component differentiated *fulvum-abyssinicum* from the remaining *Pisum* taxa, while the second discriminated between *P. fulvum* and *P. abyssinicum*. The other three *Pisum* taxa were not clearly discriminated among them. The Neighbour-Joining clustering method at the species level is shown in Fig. 4. Bootstrap values support the conclusion that *P. sativum* and *P. elatius* are sister taxa. The rest of relationships were not clearly supported by bootstrap values.

Discussion

RBIPs have proved to be suitable markers for genetic diversity evaluation, evolutionary analysis and variety discrimination in *Pisum* (Smýkal *et al.,* 2008a,b; Jing *et al.,* 2010). We have evaluated here the RBIP variability in a Spanish pea collection in relation to an accession set of the World *Pisum* core collection.

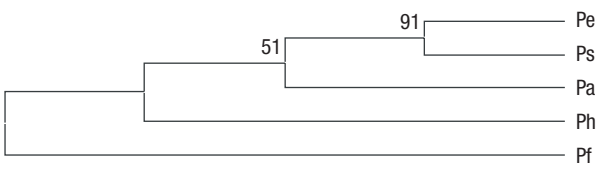

Figure 4. Neighbour joining unrooted tree at species-subspecies level. Abbreviations as in previous figures. Only bootstrap support values over 50% are indicated.

In relation to diversity at species level, the results on *P. fulvum* and *P. abyssinicum* contrast with the results described by Vershinin *et al.* (2003) using SSAP markers in which *P. fulvum, P. elatius* and *P. sativum* shared a similar high level of polymorphism, while *P. abyssinicum* showed a significant lower level of polymorphism. We suggest that these contrasting results are mainly due to the different accession sets of *fulvum* and *abyssinicum* used in both studies, and less to the markers since both RBIP and SSAP are based on retrotransposon insertion polymorphisms.

The fact that pea cultivars are grouped within a single cluster (Fig. 2, cluster 3) which represents a subset within the cultivated pea accessions set, and that their polymorphism level, estimated as H_e, is lower than pea landraces points to that only a part of the genetic variability available in traditional crop materials have been used to bred modern pea cultivars. Zong *et al.* (2009), in a comparative analysis of Chinese and global wide pea collections, stressed the importance of genetically distinct pea gene pools for future breeding programs. But even less «exotic» pea landrace collections can have additional genetic variability to be exploited in cultivar improvement. The advantage of local materials is that they are probably better adapted to the local environmental conditions than «exotic» materials. For instance, resistance to the race 6 of *Pseudomonas syringae* pv. *pisi* has been found in Spanish pea landraces (Elvira-Recuenco and Taylor, 2001; Martín-Sanz, 2008), while resistance to this race was previously only described in *P. abyssinicum* (Schmit *et al.,* 1993).

Ellis *et al.* (1998) have also described that pea cultivars, and some «landraces», formed a separate cluster from other pea materials and wild species using an almost completely different set of JI accessions from the one included here. The cluster (Ellis *et al.,* 1998) included two classes of cultivars which were bred separately as the crop has different requirements: some were bred to be harvested as immature seeds for vegetable use, and other to be harvested as dry seeds. Smýkal *et al.* (2008b) used RBIP and SSR markers to analyze a collection of 164 Czech and Slovak pea accessions, and the cluster analysis of the molecular data not fully separated fodder pea types from other pea types, and they suggested that no global genomic differences exist between the two pea types.

There was no apparent relationship among clusters and geographical origin of cultivated pea accessions, either between those from the Spanish collection or from the World collection. Previous data on a Spanish collection of 120 pea landraces indicated that the groups formed on the basis of ISSR markers were not related to agro-climatic regions within Spain (Lázaro and Aguinagalde, 2006).

Previous PCA analysis based on SSAP transposon polymorphisms (Vershinin *et al.,* 2003) have pointed out similar close relationships among *P. elatius, P. humile* and *P. sativum* to the observed in our work (Fig. 3). Likewise, clustering methods (Fig. 4) agreed with the generally accepted hypothesis that *P. fulvum* is the most distant species from cultivated *Pisum sativum* (Maxted and Ambrose, 2001; Vershinin *et al.,* 2003; Kosterin and Bogdanova, 2008). The representation of *P. humile* in our accession collection was too low to obtain valid conclusions.

Studies based in the analysis of different set of markers (retrotransposon based markers; AFLPs, mitochondrial-chloroplast-nuclear markers, etc.) agree that three main taxonomic groups of *Pisum* can be distinguished; these are *P. abyssinicum*, *P. fulvum* and *P. sativum*, other taxa once considered as species are presently considered as subspecies of *P. sativum*, although the subspecies concept in pea remains quite vague (Ellis *et al.,* 1998; Maxted and Ambrose, 2001; Kosterin and Bogdanova, 2008). Our result with RBIP markers agree with this species differentiation. Both principal component analysis and clustering method distinguished two groups including each one the *P. abyssinicum* and *P. fulvum* accessions, and clearly segregated from the remaining *Pisum* accessions. On the basis of RBIP markers, the *elatius* and *humile* accessions were scattered among other *Pisum* cluster, some near *P. abyssinicum* and most interposed with pea landraces. The same set of *P. fulvum* accessions also formed a differentiated cluster apart from other *Pisum* materials in previous works based on the use of 39 gene segments or 54 *PDR1* SSAP retrotransposon markers (Jing *et al.,* 2005; 2007). Some *P. elatius* accessions formed clusters in the phylogenetic trees described in these two previous works, while others were clustered with *P. sativum* accessions as observed in our results with RBIP markers, but in our case all *P. elatius* accessions (almost an identical set to the used by Jing *et al.,* 2007) were grouped within cluster with other *Pisum* taxa. Likewise the two *P. humile* accessions were clustered with other *Pisum* materials. In these previous works by Jing *et al.* (2005, 2007), *P. abyssinicum* was represented only by accession JI 2385, also included in this work.

Like SSAP markers (Vershinin *et al.,* 2003), all the RBIP markers analyzed here were polymorphic, with unique and species-specific markers making up only a small proportion of them. This situation is consistent with the possibility that introgression, segregation, and small rearrangements, rather than transposition itself, are the dominant modes of diversity generation in *Pisum* (Vershinin *et al.,* 2003). Likewise, according to Vershinin *et al.* (2003) the absence of common markers shared exclusively by *P. abyssinicum* and *P. sativum* strongly supports the idea that both species were brought into cultivation independently and bayesian structure analysis of RBIP data provide a plausible model for how this occurred (Jing *et al.,* 2010). Our result would support this hypothesis since, in spite that additional markers and accessions are included in the comparison, only a single common allele was shared exclusively by these two species.

The analysis of shared alleles also supported the close relationships between the *sativum* (92 accessions), *elatius* (12 accessions), and *humile* (two accessions) taxa. The numbers of alleles detected in these taxa were 50, 40 and 26, respectively. The shared alleles between *sativum* and *elatius* were 37 (over a possible maximum of 40) and between *sativum* and *humile* 23 (over a possible maximum of 26). Among these shared pairs seven were shared exclusively by *sativum* and *elatius* and one by *sativum* and *humile*, in spite of the small number of *humile* accessions considered.

Jing *et al.* (2007) pointed out that recombination has been very effective in shuffling genetic diversity between major *Pisum* lineages, and data based on transposable elements support the extensive introgression and intermixing among lineages. Even the homogeneous *P. abyssinicum* appears to have a hybrid origin (Vershinin *et al.,* 2003). And probably this is also true in relation to modern cultivars originated from the recombination and introgression of genetically distant gene pools. As consequence the use of single genetic distance to represent the genetic structure and diversity in *Pisum* is inadequate (Jing *et al.,* 2007), and this fact has implications for the management of plant genetic resources and the selection of germplasm for plant breeding. Sample pairs that are very closely related in single distance analysis may nevertheless carry distantly related gene alleles and vice versa (Jing *et al.,* 2007). This is the situation also observed in our results. For

instance, a relatively infrequent allele in locus *2055-x29* is shared by a Spanish cultivar (Ucero) and a Spanish landrace (JI1831), which differ in the alleles present in other seven loci but, on other hand, the allele is present in several cultivars or landraces from distant countries such as Canada or India. All these accessions sharing this infrequent *2055-x29* allele are scattered in cluster 3 and in other clusters of Fig. 2. A similar situation can be observed in relation to geographic information, which is also important in the design of germplasm collections. Cultivars Blizzar, Cea, Esla, Fortune, Raffale, Victor, and Iceberg and landrace ZP806 were close related by genetic distance (Fig. 2) in spite that they were bred in different countries. Thus, Jing *et al.* (2007) suggest that multilocus haplotype analysis of germplasm collections will be required to provide the solution to these problems. The importance of considering multilocus analysis in plant genetics and breeding was stressed by Allard (1999) and its importance in relation to germplasm collections was indicated (Pérez de la Vega *et al.,* 1994).

This work has provided additional information on pea germplasm collections and on the use of RBIP markers in the evaluation of genetic diversity in these collections. Data are complementary to previous data on the same or similar materials and, in general, agree and support previous conclusions on *Pisum* taxa relationships and genetic variability distribution. Results also point to that the Spanish pea core collection of landraces maintains a relatively high variability which is only partially represented in modern bred pea cultivars adapted to Spanish conditions.

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Appendix 1. *Pisum* materials used in the RBIP analysis

Accesion number/ Cultivar name	Species	Status	Country	Place	County	Complementary information[a]
JI0130	*P. abyssinicum*	Landrace	Palestine			ZP1237
JI1640	*P. abyssinicum*	Landrace	Ethiopia			ZP1246
JI2202	*P. abyssinicum*	Landrace	Ethiopia			ZP1254
JI2202/1	*P. abyssinicum*	Landrace	Ethiopia			Derived from JI2202
JI2385	*P. abyssinicum*	Landrace	Yemen			ZP1525
JI0261	*P. elatius*	Wild	Turkey			
JI1075	*P. elatius*	Wild	Turkey			
JI1094	*P. elatius*	Wild	Greece			
JI1703	*P. elatius*	Wild	Unknown			
JI2078	*P. elatius*	Wild	Unknown			
JI2105	*P. elatius*	Landrace	Iran			
JI3147	*P. elatius*	Wild	Turkey			
JI3149	*P. elatius*	Wild	Turkey			
JI3151	*P. elatius*	Wild	Turkey			
JI3155	*P. elatius*	Wild	Turkey			
JI3156	*P. elatius*	Wild	Turkey			
JI0224	*P. fulvum*	Wild	Israel			
JI1006	*P. fulvum*	Wild	Israel			
JI1010	*P. fulvum*	Wild	Iran			
JI1796	*P. fulvum*	wild	Israel			
JI2473	*P. fulvum*	Wild	Israel			
JI2517	*P. fulvum*	Wild	Syria			
JI2519	*P. fulvum*	Wild	Syria			
JI2523	*P. fulvum*	Wild	Syria			
JI2530	*P. fulvum*	Wild	Syria			
JI2544	*P. fulvum*	Wild	Syria			
JI0241	*P. humile*	Wild	Israel			
JI1794	*P. humile*	Wild	Israel			
JI0113	*P. sativum*	Cultivar	Russia			Spontaneus mutant
JI015	*P. sativum*	Cultivar	Sweden			
JI0267	*P. sativum*	Landrace	Greece			
JI0281	*P. sativum*	Landrace	Ethiopia			
JI0321	*P. sativum*	Cultivar	Canada			Alaska
JI0399	*P. sativum*	Cultivar	Netherlands			Cennia
JI0435	*P. sativum*	Cultivar	U.S.A.			Wisconsin Perfection
JI0516	*P. sativum*	Cultivar	UK			Maro
JI086	*P. sativum*	Landrace	Afghanistan			
JI0975	*P. sativum*	Landrace	Costa Rica			
JI1267	*P. sativum*	Wild	India			
JI1398	*P. sativum*	Wild	China			
JI1544	*P. sativum*	Landrace	China			
JI1775	*P. sativum*	Landrace	Chile			
JI1831	*P. sativum*	Landrace	Spain			
JI1844	*P. sativum*	Landrace	Mexico			
JI2200	*P. sativum*	Landrace	Russia			
JI2263	*P. sativum*	Wild	Tunisia			
JI2376	*P. sativum*	Landrace	Zaire			
JI2383	*P. sativum*	Landrace	Zambia			
JI2421	*P. sativum*	Landrace	Latvia			
JI2546	*P. sativum*	Wild	Georgia			*P. transcaucasicum*
JI2605	*P. sativum*	Wild	Libya			*P. speciosum*
ZP0064	*P. sativum*	Landrace	Spain		Palencia	BGE032231-CC
ZP0074	*P. sativum*	Landrace	Spain	Santibáñez de Vidriales	Zamora	BGE004041-CC

Appendix 1 (cont.). *Pisum* materials used in the RBIP analysis

Accesion number/ Cultivar name	Species	Status	Country	Place	County	Complementary information[a]
ZP0076	*P. sativum*	Landrace	Spain	Paradela del Río	León	BGE004043-CC
ZP0104	*P. sativum*	Landrace	Spain	Vall d'Alba	Castellón	BGE001034
ZP0109	*P. sativum*	Landrace	Spain	Maguilla	Badajoz	BGE001100
ZP0115	*P. sativum*	Landrace	Spain	M. de la Salud	Baleares	BGE001414-CC
ZP0126	*P. sativum*	Landrace	Spain	Jerez de los Caballeros	Badajoz	BGE001646-CC
ZP0138	*P. sativum*	Landrace	Spain	Boracan de San Cristóbal	Oviedo	BGE002088-CC
ZP0150	*P. sativum*	Landrace	Spain	Garellas	Pontevedra	BGE002165-CC
ZP0152	*P. sativum*	Landrace	Spain	Santibáñez de Vidriales	Zamora	BGE002167-CC
ZP0156	*P. sativum*	Landrace	Spain	Castiñeiro	La Coruña	BGE003046-CC
ZP0168	*P. sativum*	Landrace	Spain	Pola de Somiedo	Asturias	BGE003303-CC
ZP0171	*P. sativum*	Landrace	Spain	La Riera	Asturias	BGE003306-CC
ZP0177	*P. sativum*	Landrace	Spain	Hospital	Asturias	BGE003312-CC
ZP0180	*P. sativum*	Landrace	Spain	Llanos de Somerón	Asturias	BGE003315-CC
ZP0181	*P. sativum*	Landrace	Spain	Jomezana Baja	Asturias	BGE003316-CC
ZP0202	*P. sativum*	Landrace	Spain	Corvelle	Lugo	BGE003436-CC
ZP0206	*P. sativum*	Landrace	Spain	Corvelle	Lugo	BGE003440-CC
ZP0213	*P. sativum*	Landrace	Spain	Tagarabuena	Zamora	BGE003690-CC
ZP0344	*P. sativum*	Landrace	Spain	Cervera de Pisuerga	Palencia	BGE032241-CC
ZP0516	*P. sativum*	Landrace	Spain	Fontecha de la Peña	Palencia	BGE005515-CC
ZP0535	*P. sativum*	Landrace	Spain	Cumbres San Bartolomé	Huelva	BGE001662-CC
ZP0593	*P. sativum*	Landrace	Spain	Béjar	Salamanca	BGE001519-CC
ZP0798	*P. sativum*	Landrace	Spain	Albunan	Granada	BGE032226-CC
ZP0799	*P. sativum*	Landrace	Spain	Colomera	Granada	BGE030152-CC
ZP0806	*P. sativum*	Landrace	Spain	Alhama de Granada	Granada	BGE030158-CC
ZP1261	*P. sativum*	Landrace	Spain	Puntallana	Tenerife	BGE019598-CC
ZP1262	*P. sativum*	Landrace	Spain	Lena	Asturias	BGE019600-CC
ZP1263	*P. sativum*	Landrace	Spain	Gijón	Asturias	BGE019778-CC
ZP1264	*P. sativum*	Landrace	Spain	Pina de Ebro	Zaragoza	BGE020326-CC
ZP1278	*P. sativum*	Landrace	Spain	Zas	La Coruña	BGE023269-CC
ZP1282	*P. sativum*	Landrace	Spain	Luanco	Asturias	BGE023273-CC
ZP1290	*P. sativum*	Landrace	Spain	Zas	La Coruña	BGE023282-CC
ZP1294	*P. sativum*	Landrace	Spain	Vélez Rubio	Almeria	BGE023644-CC
ZP1300	*P. sativum*	Landrace	Spain	Peñarrubia	Cantabria	BGE024375-CC
ZP1301	*P. sativum*	Landrace	Spain	La Fuente	Cantabria	BGE024376-CC
ZP1311	*P. sativum*	Landrace	Spain	Mirandilla	Badajoz	BGE025269-CC
ZP1315	*P. sativum*	Landrace	Spain	Villamanín	León	BGE025273-CC
ZP1318	*P. sativum*	Landrace	Spain	Santiesteban del Puerto	Jaen	BGE025728-CC
ZP1338	*P. sativum*	Landrace	Spain	Torrepacheco	Murcia	BGE027119-CC
ZP1347	*P. sativum*	Landrace	Spain	Ordes	La Coruña	BGE028986-CC
ZP1355	*P. sativum*	Landrace	Spain	Xinzo de Limia	Orense	BGE028998-CC
ZP1358	*P. sativum*	Landrace	Spain	Lousame	La Coruña	BGE029002-CC
ZP1366	*P. sativum*	Landrace	Turkey			Vavilov Institute 2274
Aravalle	*P. sativum*	Breeding line	Spain			ZP1460
Baccara	*P. sativum*	Cultivar	France			ZP1457
Badmington	*P. sativum*	Cultivar	France			ZP1454
Blizzard	*P. sativum*	Cultivar	France			ZP1672
Burbia	*P. sativum*	Breeding line	Spain			ZP1664
Cea	*P. sativum*	Cultivar	Spain			ZP0866
Cheyenne	*P. sativum*	Cultivar	France			ZP1456
Chicarrón	*P. sativum*	Cultivar	Spain			ZP1666
Coomonte	*P. sativum*	Cultivar	Spain			ZP1406
Dove	*P. sativum*	Cultivar	France			ZP1667
Esla	*P. sativum*	Cultivar	Spain			ZP0864

Appendix 1 (cont.). *Pisum* materials used in the RBIP analysis

Accesion number/ Cultivar name	Species	Status	Country	Place	County	Complementary information[a]
Fortune	*P. sativum*	Cultivar	U.K.			ZP1524
Gracia	*P. sativum*	Cultivar	Spain			ZP0028
Hardy	*P. sativum*	Cultivar	France			ZP1669
Iceberg	*P. sativum*	Cultivar	Denmark			ZP1458
Ideal	*P. sativum*	Cultivar	France			ZP1463
Lobos	*P. sativum*	Breeding line	Spain			ZP1665
Lucy	*P. sativum*	Cultivar	France			ZP1670
Luna	*P. sativum*	Cultivar	Spain			ZP1231
Messire	*P. sativum*	Cultivar	France			ZP1468
Rafalle	*P. sativum*	Cultivar	France			ZP1013
Sidney	*P. sativum*	Cultivar	France			ZP1671
Speleo	*P. sativum*	Cultivar	France			ZP1673
Talanda	*P. sativum*	Breeding line	Spain			ZP1461
Ucero	*P. sativum*	Cultivar	Spain			ZP1414
Víctor	*P. sativum*	Cultivar	U.S.A.			ZP1465
Volcano	*P. sativum*	Cultivar	France			ZP1668

[a] Reference number in other germplasm bank collections. BGE: Spanish germplasm bank, these accessions are included in the Spanish pea core collection. ZP: ITACyL collection. Alaska, Cennia, Wisconsin Perfection, and Maro are cultivar names. CC: included in the Spanish core collection.

Appendix 2. RBIP markers used, primer sequences and linkage group in which they have been located

Marker name and specific primers	Annealing temperature	No occupied size (bp)	Occupied size (bp)	Linkage group
Birte-B1 5' CCCATTGATTCTCGTCTCAAGAC 5' TCACGAGGGTGTGATAGTAACTCA	55	244	281	
Birte-x16 5' CTTACCACCAAGCGCGCGAC 5' AGGCTTCTGATCCAACCAG	55	134	226	II
Birte-x34 5' GTTACTGCGGACGGTGGTC 5' GGCTGAAATCTCACTTTTGC	55	591	183	
281x1 5' TAATTATTATGGTATTCTGTG 5' CATATATTCACCCAAATCTTAAAG	55	267	240	IV
281x44 5' GATCAGAGAATCATGTCCAG 5' TCGAGGTGTGACAAAGTGC	55	339	237[a]	II
281x5 5' GTAAATATGGACGTAAGATATC 5' CGATACCCTATTCCCAAAAG	55	361	215	
1794-2 5' GGGCCATGTACGACACATTC 5' GAGGAAATAAGAATGGTAGAGCATC	55	247	181	
399-80-46 5' GTTCTACTTCCTCTGAGTCA 5' CGATACGAAGGAGGAGTTAG	55	89	167	V

Appendix 2 (cont.). RBIP markers used, primer sequences and linkage group in which they have been located

Marker name and specific primers	Annealing temperature	No occupied size (bp)	Occupied size (bp)	Linkage group
2055x29 5' CGATCATGATAAATATATTTAAT 5' CGAAGCATTAATGTATTAGAAC	55	311	183	
2055nr23 5' ATATGTGATTACGACAATAGG 5' CGACAGTGTAAATCTTTTTACA	60	256	181	
2055nr53 5' TGGATAGGGTATTGGAGTTC 5' ATAGCAGTAATTATGAACATG	55	379	434	
2385x64 5' GAAACATGATAGTAAGTTGCTC 5' CTTCCCTAAGCATTTTAATTGATC	55	556	234	
95x19 5' GGCGAGTATGTGCGCATG 5' CGACACCAGTCCCGTATTC	55	599	241	
95x2 5' CTGCAAAGGGTGCATATAG 5' GTTTTACAGGTGAAGAATCGTG	55	390	213	VI
64x45 5' CAAAGTATAACGTGTATCAAG 5' GTCATCGTCCAAACACACTC	55	641	601[a]	
45x15 5' CGAAGTCAATATAATTGGCG 5' TCAACATGTCACTCCCATTAC	55	320	285	III
45x29 5' TGATGAACAGCATCCTGG 5' CGAAACTGGCTAGTTGCAAG	55	412	197	VII
45x31 5' GACTACTAGTTGGAACTCTTG 5' CATCTGGTTAGACAAGAAGAG	60	289	340[a]	II

[a] These bands when the retrotransposon is inserted were obtained with the general primer 5' GGGCTTTGACTAATGGACCTC, the rest were obtained with the general primer 5' TAAGGTCCATTAGTCAAAGCCC.